Jpn. J. Cancer Res. **93**, 42–49, January 2002

Chemopreventive Effects of a Flavonoid Antioxidant Silymarin on *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine-induced Urinary Bladder Carcinogenesis in Male ICR Mice

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The modifying effects of dietary administration of a flavonoid antioxidant, silymarin, a mixture of three flavonoids isolated from milk thistle seeds, on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN)-induced urinary bladder carcinogenesis were examined in male ICR mice. Animals were divided into 5 groups, and groups 1 to 3 were given OH-BBN (500 ppm) in drinking water for 6 weeks. Mice in group 2 were fed a diet containing 1000 ppm silymarin for 8 weeks during the initiation phase starting 1 week before OH-BBN exposure, and mice in group 3 were fed the diet for 24 weeks during the postinitiation phase. Animals in group 4 were given only the test compound, and those in group 5 were given the basal diet alone throughout the experiment. Animals were sacrificed at the end of week 32. The frequency of bladder lesions, cell proliferation and cell cycle progression activity estimated in terms of the 5-bromodeoxyuridine (BrdU) labeling index or cyclin D1-positive cell ratio were compared among the groups. Administration of silymarin in the initiation or postinitiation phase significantly decreased the incidences of bladder neoplasms and preneoplastic lesions. Dietary exposure to this agent significantly reduced the labeling index for BrdU and the cyclin D1-positive cell ratio in various bladder lesions. These findings suggest that silymarin is effective in preventing OH-BBN-induced bladder carcinogenesis in mice.

Key words: Chemoprevention — Silymarin — OH-BBN — Cyclin D1 — Urinary bladder carcinogenesis

The incidence of bladder cancer has been increasing in most industrialized countries.[1)] In the United States, 50 500 new cases of the disease were recognized in 1995.[2)] In the light of the high incidence, increasing attention has been focused on the prevention of bladder cancer at the earliest possible stage.

A recent approach to curb cancer incidence is chemopreventive intervention, through which the disease can be prevented totally, or slowed, or reversed partially or substantially by the administration of one or more naturally occurring or synthetic chemical agents. Fruit, vegetables, and beverages, as well as several herbs and plants with diverse pharmacological properties, have been shown to be rich sources of microchemicals with potential to prevent the occurrence of cancers.[3–7)] The chemopreventive potential of flavonoids in various organs has been established *in vivo* as well as *in vitro*.[8–10)]

Among flavonoids, silymarin, a mixture of three structural isomers (Fig. 1) is known to be present in milk thistle (*Silybum marianum*) seeds and is used clinically in Europe, Asia, and other countries basically for the treatment of liver disease.[11–13)] It is well known that silymarin is capable of scavenging both free radicals and reactive oxygen species in rodents and in cell cultures.[14–17)] This agent is reported to abolish the lethal effects and pathological changes induced by microcystin-LR intoxication.[18)] In human studies, it was shown that serum transaminases and sulfobromophthalein retention in the silymarin-treated group displayed a significantly greater tendency to normalization.[19)] Tumor-preventive effects of silymarin and its major component, silybin (or silibinin), have received attention in both *in vivo* and *in vitro* models. In particular, exposure to silymarin resulted in a highly significant protection against benzoyl peroxide (BPO)-induced tumor promotion in 7,12-dimethylbenz[*a*]anthracene-initiated skin carcinogenesis in SENCAR mice.[20)] This agent is also reported to inhibit cell proliferation, cell growth, and cell cycle progression in various types of cancer.[21–24)]

In the present study, chemopreventive effects of silymarin were examined by exposure during the initiation and postinitiation stages on *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (OH-BBN)-induced urinary-bladder carcinogenesis in mice. In addition, effects of silymarin on cell cycle progression as well as cell proliferation activities in the bladder epithelium of mice exposed to OH-BBN were examined by analyzing ratio of cells positive for cyclin D1 and the 5-bromodeoxyuridine (BrdU) labeling index (LIs).

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MATERIALS AND METHODS

Animals, diet and chemicals A total of 147 male ICR mice, were obtained from Japan SLC, Inc., Hamamatsu. OH-BBN was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo. Powdered CE-2 (CLEA Japan, Inc., Tokyo) was used as a basal diet. Silymarin was purchased from Sigma (St. Louis, MO). Experimental food mixed with the test compound was prepared twice a week. OH-BBN was given to mice in tap water at a concentration of 500 ppm. The drinking water containing carcinogen was prepared every other day. All animals were allowed free access to food and water. Animals were housed in plastic cages (5 per cage) with wood chips in an air-conditioned room at 23±2°C (SD), 50±10% relative humidity, under a 12-h light/dark cycle.

Experimental procedure Five-week-old mice were randomly divided into 5 groups (Fig. 2). After a 1-week quarantine, animals in groups 1–3 (44 mice, 34 mice and 30 mice, respectively) received OH-BBN in the drinking water for 6 weeks. Mice in group 2 were given the diet containing 1000 ppm silymarin starting 1 week before OH-BBN exposure until the end of week 8, then they were maintained on the basal diet for the subsequent 24 weeks. Group 3 was fed food mixed with silymarin starting 1 week after cessation of the OH-BBN treatment and kept on this diet until the termination. Group 4 (15 mice) was not exposed to the carcinogen and was fed the diet with the test compound throughout the experiment. Group 5 (24 mice) was given the basal diet and tap water without OH-BBN throughout the experiment, serving as an untreated control. All mice were weighed once a week for the first 8 weeks, and once a month for the subsequent period. The experiment was terminated at 32 weeks, and all animals were killed under ether anesthesia. For measurement of BrdU-incorporating nuclei, BrdU (Sigma-Aldrich Co., Ltd., Tokyo) solution was injected i.p. at a dose of 50 mg/kg body weight 1 h before death. At autopsy, the urinary bladder was fully inflated with 10% buffered formalin, fixed for 5 h and embedded in paraffin for histopathological evaluation on hematoxylin and eosin-stained sections according to the criteria described by Fukushima *et al.*[25] Other organs including liver, kidney, lungs, stomach and

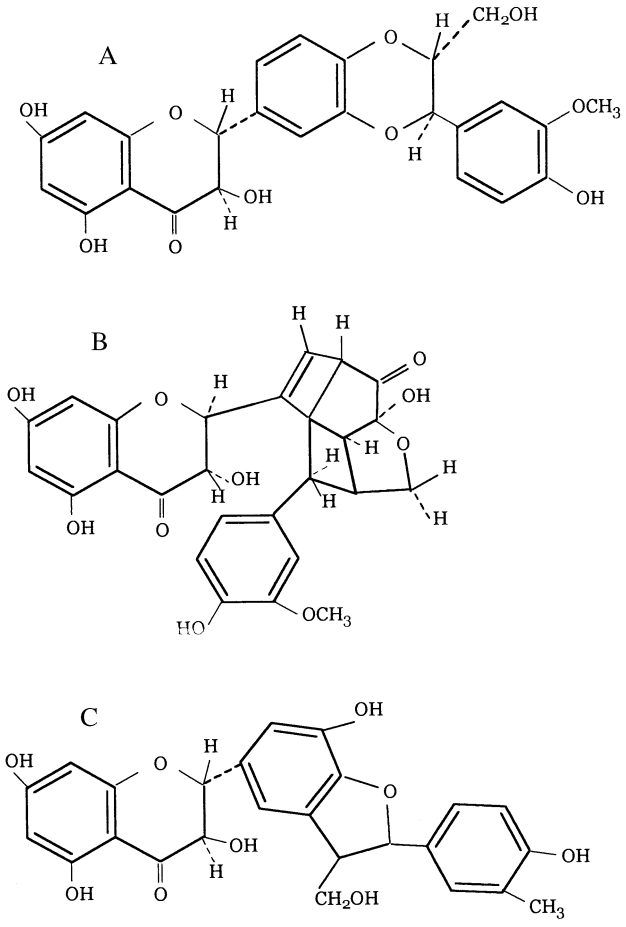

Fig. 1. Chemical structures of (A) silibinin (or silybin), (B) silydianin and (C) silychristin.

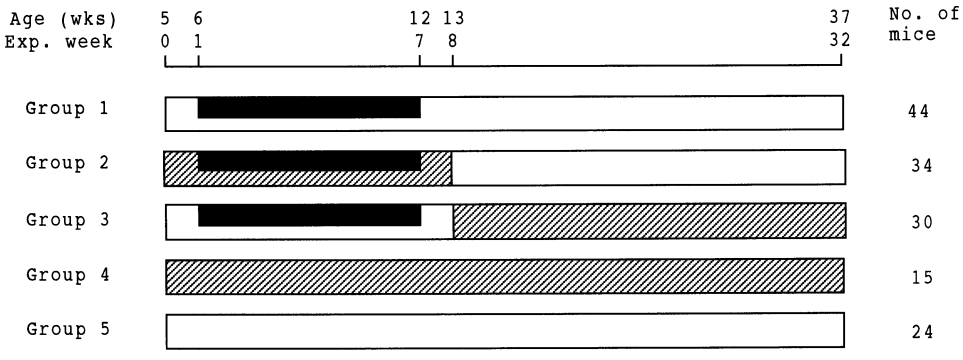

Fig. 2. Experimental protocol. ■ 500 ppm OH-BBN in drinking water, ▨ 1000 ppm silymarin, □ basal diet and tap water.

Table I. Body, Liver and Relative Liver Weights at the End of the Study

Group	Treatment	No. of mice	Body wt. (g)	Liver wt. (g)	Relative liver wt. (g/100 g liver wt.)
1	OH-BBN	44	50.2±3.6[a, b]	2.9±0.52[c]	5.75±0.5
2	OH-BBN+Silymarin	34	53.5±3.2	3.0±0.48	5.63±0.8
3	OH-BBN→Silymarin	30	51.0±6.6	3.1±0.52	6.07±1.0
4	Silymarin	15	54.1±7.2	3.2±0.46	5.87±0.4
5	Vehicle control	24	54.9±5.8	3.2±0.48	5.69±0.5

a) Mean±SD.
b, *c*) Significantly different from group 5 by Student's *t* test (*b*) $P<0.005$, *c*) $P<0.05$).

Table II. Incidence of Tumors in the Bladder of Mice

Group	Treatment	No. of mice examined	No. of mice (%) with	
			Papilloma	TCC[a]
1	OH-BBN	44	2 (5)	17 (39)
2	OH-BBN+Silymarin	34	1 (3)	4 (12)[b]
3	OH-BBN→Silymarin	30	5 (17)	5 (17)[c]
4	Silymarin	15	0 (0)	0 (0)
5	Vehicle control	24	0 (0)	0 (0)

a) TCC, transitional cell carcinoma.
b, *c*) Significantly different from group 1 by Fisher's exact probability test (*b*) $P<0.01$, *c*) $P<0.05$).

intestine were fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by conventional histological methods using hematoxylin and eosin staining.

Immunohistochemical staining For the evaluation of cell proliferation and cell cycle activity of the epithelial cells, BrdU and cyclin D1 immunohistochemistry was performed according to the methods described previously with some modifications.[26, 27] Briefly, 3-μm paraffin-embedded sections were deparaffinized with three changes of xylene and hydrated using a graded series of alcohol. Before staining, slides for cyclin D1 immunohistochemistry were incubated in 1 m*M* EDTA (pH 8.0) at 121°C twice in an autoclave, 5 min each to effect antigen retrieval, then exposed overnight to 1:100 diluted cyclin D1 mouse monoclonal antibody (Novocastra Laboratories, Newcastle upon Tyne, UK), thereafter slides were developed by the avidin-biotin-peroxidase complex method. Slides for BrdU immunohistochemistry were incubated in 3% hydrogen peroxide at room temperature for 30 min to block endogenous peroxidase and then tissue sections were treated with 2 *N* HCl at room temperature for 20 min and neutralized with Tris-buffered saline. Subsequently, they were incubated with 0.02% actinase E (Kaken, Tokyo) at 37°C for 20 min. After incubation with normal horse serum at room temperature for 30 min, they were exposed overnight at 4°C to mouse monoclonal antibody to BrdU (Amersham Pharmacia Biotech, Buckinghamshire, UK) diluted 1:100. The subsequent steps were the same as those for cyclin D1 staining. Cells were considered positive for cyclin D1 or BrdU when definite nuclear staining was identified. To score each parameter, the positive cell ratio for cyclin D1 (or labeling indexes (LIs) for BrdU) was determined by randomly observing 500 epithelial cells under ×400 magnification (over 50 fields). Positive cell ratios (or LIs) were calculated as numbers per 100 cells. Overexpression of cyclin D1 in preneoplastic lesions and tumors was defined as positive when nuclear staining of >5% of nuclei was evident.[28]

Statistical analysis Body weight, liver weight, relative liver weight, the incidence of lesions or BrdU-LIs and cyclin D1-positive cell ratios were compared between groups using Fisher's exact probability test, the χ^2 test, Welch's test or Student's *t* test. The results were considered statistically significant if $P<0.05$.

RESULTS

General observations There were no clinical signs of toxicity, low survival, poor condition or histological changes suggesting toxicity in the liver, kidney and lung caused by administration of the experimental diet. Mean body, liver and relative liver weights (g/100 g body wt.) in all groups at the end of the study are shown in Table I. The average body weights in group 1 were significantly smaller than those of group 5 ($P<0.005$). The average liver weights of group 1 were significantly less than those of group 5 ($P<0.05$). Mean intake of food was constant and similar among the groups during the whole experimental period (4.78–5.26 g/day/mouse). Mean intake of OH-BBN (groups 1, 2 and 3) was 0.205–0.236 mg/day/mouse).

Incidence of tumors and preneoplastic lesions of urinary bladder Tumors developed only in urinary bladders of mice in OH-BBN-treated groups (groups 1 through 3). Macroscopically, no abnormal lesions were found in other organs. Incidences of tumors are summarized in Table II. In group 1, bladder papillomas and carcinomas were recognized in 2/44 mice (5%) and 17/44 mice (39%), respec-

tively. Incidences of carcinoma of groups 2 and 3 were smaller than that of group 1 ($P<0.01$ and $P<0.05$, respectively). The incidences of preneoplastic lesions in all groups are indicated in Table III. The incidences of papillary and nodular hyperplasia of groups 2 and 3 were lower than that of group 1 ($P<0.002$ and $P<0.01$ respectively), and those of dysplasia of the two groups were also lower than that of group 1 ($P<0.01$ and $P<0.03$ respectively). In our experiment, a few squamous cell carcinomas were found in groups 1–3 (2 in group 1, 1 in group 2 and 1 in group 3) and no significant difference was observed among groups. No abnormalities were found microscopically as well as macroscopically in mice without OH-BBN treatment (groups 4 and 5).

Cyclin D1-positive cell ratios The intensity of cyclin D1 staining was generally strong among the various lesions of the urinary bladder, and was weak in simple hyperplasias and papillary or nodular hyperplasias. As summarized in

Table III. Incidence of Preneoplastic Lesions in the Bladder of Mice

Group	Treatment	No. of mice examined	No. of mice (%) with			
			Hyperplasia			Dysplasia
			Total	SH[a]	PN[a]	
1	OH-BBN	44	43 (98)	39 (88)	30 (68)	30 (68)
2	OH-BBN+Silymarin	34	31 (91)	31 (91)	11 (32)[b]	13 (38)[c]
3	OH-BBN→Silymarin	30	26 (87)	26 (87)	11 (37)[c]	12 (40)[d]
4	Silymarin	15	0 (0)	0 (0)	0 (0)	0 (0)
5	Vehicle control	24	0 (0)	0 (0)	0 (0)	0 (0)

a) SH, simple hyperplasia; PN, papillary or nodular hyperplasia.
b, *c*, *d*) Significantly different from group 1 by χ^2 test (*b*) $P<0.002$, *c*) $P<0.01$, *d*) $P<0.03$).

Table IV. Cyclin D1-positive Ratio (%) in the Normal Transitional Epithelium and Various Lesions of the Bladder

Group	Treatment	Normal epithelium	Non-lesional areas	HP[a]	DYS[a]	PAP[a]	TCC[a]
1	OH-BBN	ND[a]	4.12±0.84[b]	19.2±4.8	30.6±3.5	45.1±5.3	55.9±12.5
2	OH-BBN+Silymarin	ND	3.26±1.14[c]	16.6±2.8[c]	19.0±2.1[d]	29.9±1.6[e]	43.6±4.2[f]
3	OH-BBN→Silymarin	ND	2.12±0.12[g]	16.5±5.0	15.4±3.7[c]	30.3±3.8[e]	44.4±7.3[f]
4	Silymarin	0.35±0.44	ND	ND	ND	ND	ND
5	Vehicle control	0.35±0.82	ND	ND	ND	ND	ND

a) HP, hyperplasia; DYS, dysplasia; PAP, papilloma; TCC, transitional cell carcinoma; ND, not determined.
b) Mean±SD.
c, *d*, *f*) Significantly different from group 1 by Welch's test (*c*) $P<0.01$, *d*) $P<0.03$, *f*) $P<0.005$).
e, *g*) Significantly different from group 1 by Student's *t* test (*e*) $P<0.005$, *g*) $P<0.0001$).

Table V. BrdU-labeling Index (%) in the Normal Transitional Epithelium and Various Lesions of the Bladder

Group	Treatment	Normal epithelium	Non-lesional areas	HP[a]	DYS[a]	PAP[a]	TCC[a]
1	OH-BBN	ND[a]	0.52±1.28[b]	2.18±0.06	4.26±2.62	4.04±1.99	31.46±7.29
2	OH-BBN+Silymarin	ND	0.53±1.31	1.89±0.24	0.45±2.80[c]	0.10±0.80[d]	18.08±3.55[e]
3	OH-BBN→Silymarin	ND	0.44±0.48	1.99±0.18	0.48±2.00[c]	0.48±2.08[d]	15.01±5.69[f]
4	Silymarin	0.48±0.70	ND	ND	ND	ND	ND
5	Vehicle control	0.49±0.77	ND	ND	ND	ND	ND

a) HP, hyperplasia; DYS, dysplasia; PAP, papilloma; TCC, transitional cell carcinoma; ND, not determined.
b) Mean±SD.
c, *d*) Significantly different from group 1 by Student's *t* test (*c*) $P<0.05$, *d*) $P<0.001$).
e, *f*) Significantly different from group 1 by Welch's test (*e*) $P<0.05$, *f*) $P<0.03$).

Table IV, cyclin D1 overexpression was observed in various types of lesions. Cyclin D1 positive ratios of the nonlesional areas of groups 2 and 3 and that of hyperplasia of group 2 were respectively smaller than those of group 1 ($P<0.01$, $P<0.0001$) and ($P<0.01$). Cyclin D1-positive ratios of dysplasia of groups 2 and 3 were significantly smaller than that of group 1 ($P<0.03$ and $P<0.01$ respectively). For both papilloma and transitional cell carcinoma, cyclin D1-positive ratios of groups 2 and 3 were significantly smaller than that of group 1 ($P<0.005$).

BrdU-LIs Data on BrdU-LIs are summarized in Table V. BrdU-LIs of dysplasia of both groups 2 and 3 were significantly lower than that of group 1 ($P<0.05$). Similarly, BrdU-LIs of papilloma of both groups 2 and 3 were significantly lower than that of group 1 ($P<0.001$). BrdU-LIs of carcinoma of groups 2 and 3 were significantly lower than that in group 1 ($P<0.05$ and $P<0.03$, respectively).

DISCUSSION

The present study has confirmed a chemopreventive efficacy of flavonoid silymarin, on OH-BBN-induced urinary bladder carcinogenesis in mice.

Silymarin has been reported to suppress the growth of human cancer cells by (a) perturbation of cell cycle progression leading to G1 arrest in a dose- and time-dependent manner and (b) inhibiting DNA synthesis, possibly due to an effect of G1 arrest.[22, 23, 29, 30] Silymarin is also known to exert an antipromoting effect on skin tumorigenesis in mice mediated by impairment of receptor and nonreceptor tyrosine kinase signaling pathways.[31]

Cyclin D1 is a member of the G1 cyclin family which is involved in regulating the transition through the G1 phase of the cell cycle.[32, 33] Cyclin D1 overexpression has been reported in various human malignant tumors[34–38] and in murine chemical carcinogenesis.[39–42] Overexpression of cyclin D1 is suggested to be associated with BBN-induced urinary bladder carcinogenesis, that is, overexpression was higher in the order simple hyperplasia, papillary or nodullar hyperplasia, papilloma and carcinoma.[43, 44] In transitional carcinomas of human urinary bladder, a significant relationship between cyclin D1 overexpression and tumor grade or stage has been reported.[28]

In this study, the intensity of cyclin D1 overexpression in mice treated with OH-BBN alone was basically in the order of hyperplasia, dysplasia, papilloma and carcinoma. However, such immunoreactivity of cyclin D1 was not always analogous to that of BrdU-LIs, which was in the order of hyperplasia, papilloma, dysplasia and carcinoma. This was in line with previous studies reporting no correlation between the level of cyclin D1 expression and cell-proliferation marker (proliferating cell nuclear antgen, PCNA) expression or the number of nucleolar organizer regions in esophageal carcinoma cell lines.[45, 46] Our results imply that the expression of cyclin D1 is not simply a direct consequence of increased cell proliferation in rodent tumors.[39, 40, 47, 48] Zi *et al.* however, reported a strong antiproliferative effect of silymarin in human breast and prostate carcinoma cells. They suggested that cell cycle-regulatory proteins and associated kinase activity are relevant to the possible molecular mechanism of the effect of silymarin in two ways: first there is a significant increase in Cip 1/p21 expression that leads to its increased binding with cyclin-dependent kinase 2 (CDK2), cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6), resulting in a marked decrease in their kinase activity, and second, there is a decrease in G1 cyclin D1.[22, 29] The study also suggested that this effect is likely to involve impairment of the erbB1-Shc-ERK1/2-mediated mitogenic signaling pathway, leading to induction of CDKIs that inhibit the growth-promoting activity of CDKs, causing G1 arrest followed by cell growth inhibition, including inhibition of cyclin D1.[29] Silymarin is also reported to result in a highly significant decrease of the kinase activity of CDK6 and cyclin D1 in a time-dependent manner in human epidermoid carcinoma cells.[24] These findings suggest that the effect of silymarin is initially mediated via its effect on the kinase activity of cyclin-dependent kinases and cyclin D1.

In not only postinitiation, but also the initiation phases, chemopreventive effects of silymarin may be related to inhibition of cyclin D1, since the cell cycle has been indicated to play an important role in the initiation phase of carcinogenesis.[49, 50] There may also be another pathway of inhibition at this stage. The possible mechanism of chemoprevention by flavonoids have been suggested to involve the inhibition of the formation of the ultimate carcinogen by inhibiting the cytochrome P450 monooxygenase system.[51] We hypothesized that silymarin could inhibit the metabolic activation of OH-BBN in the initiation stage, as silymarin is known to inhibit the P450-dependent monooxygenases,[52–54] which can activate many pro-carcinogens, including *N*-nitrosamines.[55]

In our study, silymarin (1000 ppm in diet) significantly reduced the incidence of preneoplastic and neoplastic lesions when given during the initiation and postinitiation phases. The concentration of 1000 ppm was selected since striking preventive effects at this dose had been observed in our previous studies (data not shown). The results of the present immunohistochemistry analysis imply that the effect of silymarin is related to inhibition of cyclin D1. Other selective cyclin D1 inhibitors such as mandarin juices rich in β-cryptoxanthin, hesperidine and inostamycin have also been reported to possess chemopreventive potential against colon carcinogenesis in rats.[56]

Silymarin is a mixture of three structural isomers of flavonoids: silibinin (or silybin), silydianin and silychristin. The former is suggested to be the most active constituent. Since the cancer chemopreventive and anti-carcinogenic

effects of silymarin appear to be due to the main constituent silibinin,[30] further studies of the chemopreventive effects of silibinin itself are necessary.

In conclusion, our study clarified the chemopreventive effect of silymarin on urinary bladder carcinogenesis in an animal model. This compound could be a promising agent for prevention of bladder neoplasia.

ACKNOWLEDGMENTS

We would like to thank K. Takahashi and C. Usui for technical assistance, and A. Nagata and T. Kajita for secretarial assistance.

(Received August 6, 2001/Revised November 1, 2001/Accepted November 7, 2001)

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